

## Apical Membrane Antigen 1, a Major Malaria Vaccine Candidate, Mediates the Close Attachment of Invasive Merozoites to Host Red Blood Cells

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**Apical membrane antigen 1 (AMA-1) of *Plasmodium* merozoites is established as a candidate molecule for inclusion in a human malaria vaccine and is strongly conserved in the genus. We have investigated its function in merozoite invasion by incubating *Plasmodium knowlesi* merozoites with red cells in the presence of a previously described rat monoclonal antibody (MAb R31C2) raised against an invasion-inhibitory epitope of *P. knowlesi* AMA-1 and then fixing the material for ultrastructural analysis. We have found that the random, initial, long-range (12 nm) contact between merozoites and red cells occurs normally in the presence of the antibody, showing that AMA-1 plays no part in this stage of attachment. Instead, inhibited merozoites fail to reorientate, so they do not bring their apices to bear on the red cell surface and do not make close junctional apical contact. We conclude that AMA-1 may be directly responsible for reorientation or that the molecule may initiate the junctional contact, which is then presumably dependent on Duffy binding proteins for its completion.**

The pathology of malaria is a consequence of the parasitemia which develops through cyclical asexual replication of *Plasmodium* sp. parasites in a patient's red blood cells (RBC). The malaria parasite's ability first to recognize and then to invade RBC is central to the disease process, and parasite molecules involved in these recognition and invasion steps are widely agreed to be targets for prophylactic immunization. (For recent reviews of the pathogenesis of malaria and of the prospects for immunization, see references 24 and 32.) A prime candidate immunogen for inclusion in a human *Plasmodium falciparum* malaria vaccine is apical membrane antigen 1 (AMA-1) (12, 21, 28), a protein of the extracellular, RBC-invading merozoite stage.

The AMA-1 molecule of *P. falciparum* (PfAMA-1) is synthesized as an 83-kDa precursor, from which an N-terminal prodomain is cleaved (21, 27). The mature 66-kDa form of PfAMA-1 is found in micronemes, organelles of the merozoite apical complex (7, 20). It has the attributes of an integral membrane protein, with a short cytoplasmic sequence at its C terminus and three disulfide-constrained domains forming its ectodomain. Further processing results in the shedding of fragments of 44 or 48 kDa and occurs in association with the relocation of the molecule to the merozoite surface (21, 27). PkAMA-1 from *Plasmodium knowlesi* was the first AMA-1 family protein to be recognized (12, 14). Although this malaria parasite does not synthesize a large AMA-1 precursor, the AMA-1 molecules of the two species are essentially colinear

downstream of the prosequence and the origin and fate of the PkAMA-1 molecule seem otherwise to be similar to those of PfAMA-1 (33, 39).

The candidacy of AMA-1 for inclusion in a malaria vaccine is supported by evidence drawn from studies of malaria in several animal model systems and in humans. To summarize, cognate antibody is inhibitory to parasite multiplication in vitro, while interacting with the merozoite surface (34), and also in vivo (26). AMA-1 has conferred protection in active immunization studies (11, 13, 26). Analysis of immune responses in a community in Kenya naturally exposed to *P. falciparum* has suggested that the recognition of T-cell epitopes in the molecule is rather labile but that the response to at least one epitope is associated with a lowered risk of disease (37).

Some of the data discussed above point to a role for AMA-1 in RBC invasion. This interpretation is supported by, first, the finding in vitro for *P. knowlesi* that antibodies inhibit RBC invasion by free merozoites (35) and that these antibodies do not mediate inhibition by merozoite agglutination (32). Second, it has been suggested that PfAMA-1-derived peptides inhibit merozoite interaction with RBC (38), and third, it has been suggested that there is a broadening of the range of species of target RBC which can be invaded by parasites whose AMA-1 gene has been complemented by a heterospecific AMA-1 (36). Fourth, COS-7 cells transfected with *Plasmodium yoelii* AMA-1 bind RBC (17), and finally, a peptide selected from a random phage display library by its high affinity for PfAMA-1 is a potent inhibitor of merozoite invasion (22). RBC invasion by malaria parasites is complex and involves several discernible stages (for reviews, see references 4 and 29). The merozoite's initial attachment to an RBC surface is random (16), is reversible, and may be mediated by major

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components of the complex merozoite coat-forming filamentous attachments between the two membranes at a distance of about 12 nm (9), although the receptors and ligands involved remain incompletely defined in molecular terms. This early "distant" attachment can considerably deform the host cell, giving an appearance of its wrapping around the merozoite (25) in a series of oscillations (16), but such attachment is followed by a crucial event, the reorientation of the parasite to bring its apical prominence into contact with the host cell. After apical orientation there is a tight interaction between parasite and host cell membranes, forming a junctional zone marked by a dense undercoating of the RBC membrane (23) and a closer intermembrane distance of about 4 nm (9).

Following this apical attachment, secreted material from apical organelles produces local vacuolation of the RBC membrane and the generation of an invagination, the invasion pit, beneath the region of contact (8). The merozoite now moves into the deepening invagination, apparently employing an actin-myosin motor (30) to drive the electron-dense junction as an annulus of close contact rearwards over its surface (3). The membrane of the invagination eventually seals over at the posterior of the merozoite to form the parasitophorous vacuole. For technical reasons the most complete ultrastructural descriptions of invasion obtained so far have been from *P. knowlesi*, the species which we have used in the present experiments. It is clearly desirable to understand the functions of candidate vaccine molecules wherever possible, and here we report evidence for the mode of action of an antibody against PkAMA-1 which inhibits invasion and which also suggests a precise biological function for AMA-1 in the invasion process.

#### MATERIALS AND METHODS

Monoclonal antibody (MAb) R31C2, which recognizes *P. knowlesi* AMA-1 and inhibits invasion, and control noninhibitory MAb R12C3 (12) (all rat) were used as purified immunoglobulin G (IgG) (34). *P. knowlesi* merozoites were prepared from previously cryopreserved samples of infected rhesus monkey (*Macaca mulatta*) blood, using a brief maturation culture followed by syringe release from mature schizonts as previously described (8, 19). Free merozoites were immediately added in numerical excess to Eppendorf tubes containing 2% (vol/vol) suspensions of rhesus monkey RBC in RPMI 1640 (Gibco) supplemented with 10% homologous serum which had been decomplexed by prior heating at 56°C for 30 min. Cultures contained 2 mg of IgG · ml<sup>-1</sup>. They were briefly incubated at 37°C and after 10 or 50 min fixed by addition of excess 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) before processing for electron microscopy (EM) as described previously (8). Further control samples were incubated without antibody and fixed 1 min after mixing merozoites and RBC.

#### RESULTS

In all preparations, some RBC infected with mature parasites from the first generation could be found. In control preparations, where released merozoites were incubated with RBC either without antibody or in the presence of noninhibitory MAb, electron microscopy showed that invasion was essentially complete after 10 min. At 50 min ring stage parasites were well advanced in development (Fig. 1). A few extracellular, unattached, damaged merozoites remained.

Preparations incubated in the presence of 2 mg of inhibitory MAb R31C2 · ml<sup>-1</sup> were strikingly different. After 50 min, merozoites were still extracellular and attached by their non-apical surfaces to RBC. They were rounded and undergoing degeneration (Fig. 2). Invasion was inhibited. After extensive

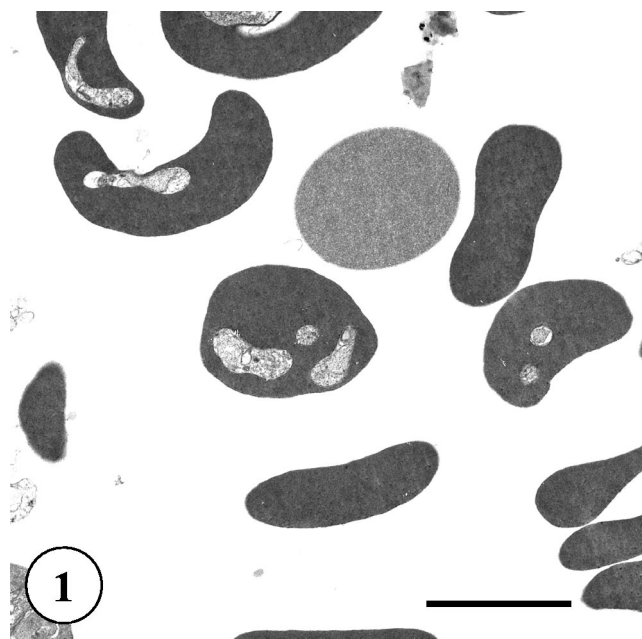


FIG. 1. General survey of normal invasion by *P. knowlesi* 50 min after addition of merozoites to fresh RBC, showing a number of ring stages. Scale bar, 5  $\mu$ m.

searches through sectioned material in replicate experiments performed on separate occasions, we could not find any invaded merozoites. Examination of material fixed at 10 min incubation with R31C2 showed that many merozoites had made initial random attachments but that the normal reorien-

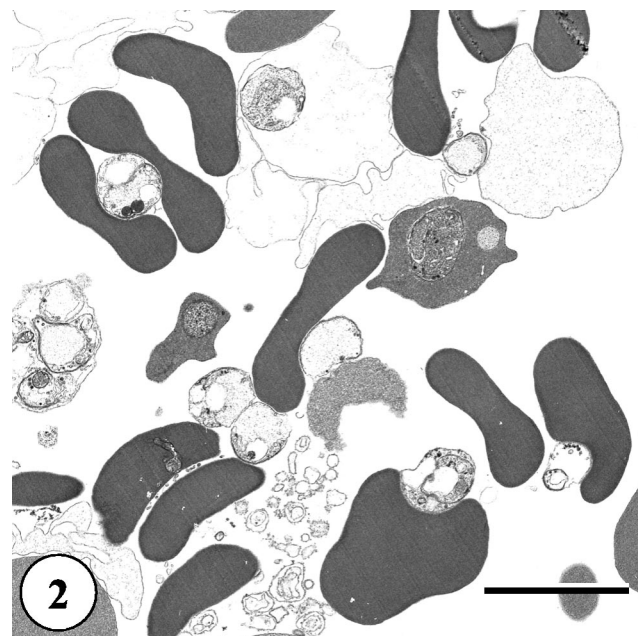


FIG. 2. General survey of a merozoite-RBC culture incubated for 50 min in the presence of inhibitory antibody R31C2, showing attachment of merozoites and absence of ring stages. RBC infected with mature parasites were seen. These derive from the first generation, used to prepare the merozoite inoculum. Scale bar, 5  $\mu$ m.

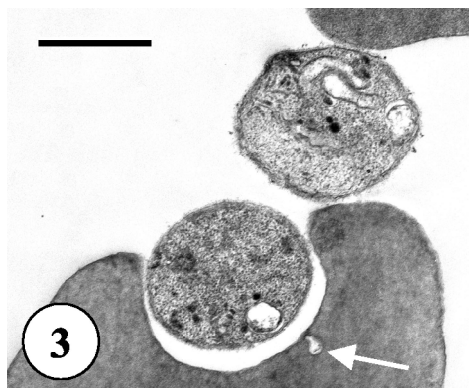


FIG. 3. Examples of merozoites attached to RBC by their nonapical surfaces at 10 min of incubation in the presence of inhibitory antibody R31C2. Note a small membranous vacuole present in the RBC (arrow), indicating transient apical contact by a merozoite. The RBC is partially wrapped around the lower merozoite, but there is no close junctional attachment. Scale bar, 1  $\mu$ m.

tation of merozoites had not occurred. Nonapical regions of the merozoite were involved. Merozoite structure appeared to be normal, with micronemes, rhoptries, and dense granules still visible. In some sections, induced RBC deformation had resulted in the partial wrapping of some parasites by the RBC (Fig. 3 and 4), but this was clearly distinct from invasion. These features have been reported for the early stages of attachment by normally invading *P. knowlesi* merozoites in the absence of antibody (5, 6, 9) and were seen in our controls fixed at 1 min (Fig. 5). Detailed examination showed that contact was of the distant type (12 nm, membrane to membrane), involving merozoite coat filaments (Fig. 6 and 7), and unlike the close junctional contact seen at the apex in normal uninhibited invasion (Fig. 8). Interestingly, a few RBC showed small irregular vacuoles in their superficial cytoplasm, similar to those occasionally seen in the early stages of normal invasion (Fig. 3) (3, 5).

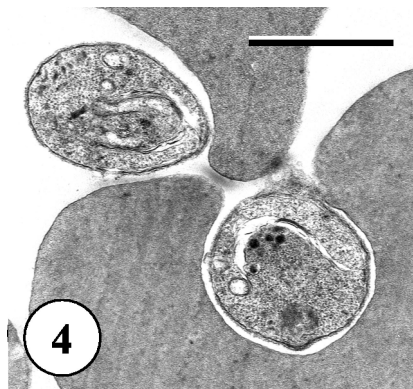


FIG. 4. Another example of nonapical attachment in the presence of inhibitory antibody (10 min). Again, although the lower merozoite gives the superficial appearance of undergoing invasion, there is no close junction, and the RBC is partially wrapped around the merozoite, as also seen in the early stages of normal invasion (see Fig. 5). Scale bar, 1  $\mu$ m.

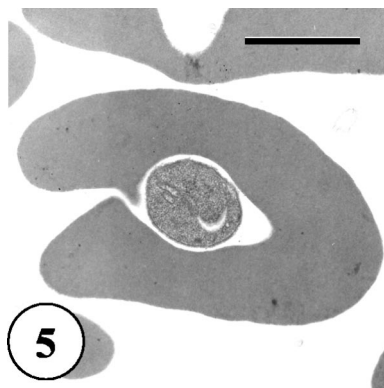


FIG. 5. An example of early merozoite-RBC interaction in normal invasion (1 min) in which the RBC is wrapped around the merozoite without forming a close junctional contact. Scale bar, 2  $\mu$ m.

## DISCUSSION

Our EM results show that the initial random surface attachment of merozoites to RBC, mediated by filaments of the merozoite's coat, occurs despite the presence of inhibitory levels of R31C2 IgG. Stages were seen which superficially resemble, but were distinct from, the internalization of merozoites; these result from the oscillatory wrapping of the merozoite by the RBC (16). We can deduce that neither the general distant interaction nor the mediation of this wrapping depend on AMA-1. However, no evidence was found of close apical junction formation by merozoites in any of the EM sections examined, so it is clear that merozoites could not reach this point in the invasion process in the presence of inhibitory antibody to AMA-1. Junction formation is essential for the further steps in invasion, and apical reorientation necessarily precedes junction formation.

It has recently been shown that PfAMA-1 is a micronemal component in the maturing merozoite (7, 20) and is strongly associated with the periphery of micronemes (7). The molecule has the characteristics of an integral membrane protein (21) and may therefore be inserted in the micronemal bounding membrane. No mechanism for its insertion into the merozoite

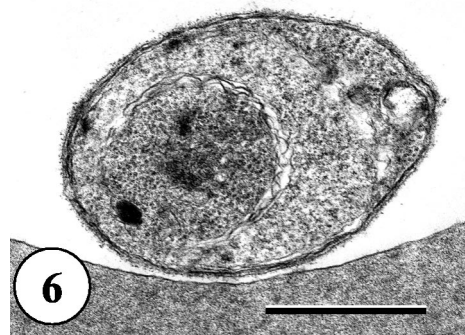


FIG. 6. Distant merozoite-RBC attachment in the presence of inhibitory antibody R31C2 (10 min of incubation), the merozoite adhering to the RBC membrane by means of the filamentous merozoite coat (FC), as shown in detail (Fig. 7). Scale bar, 1  $\mu$ m.



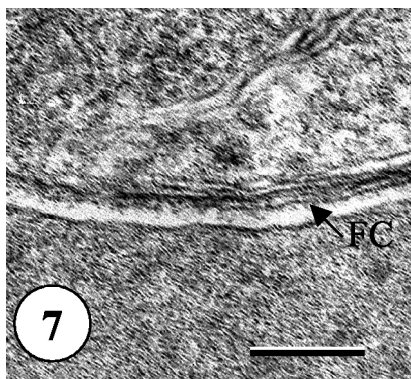


FIG. 7. Distant merozoite-RBC attachment in the presence of inhibitory antibody R31C2 (10 min of incubation), the merozoite adhering to the RBC membrane by means of the filamentous merozoite coat (FC). Scale bar, 0.5  $\mu$ m.

plasma membrane is yet resolved, but it may flow out with the micronemal membrane directly into the merozoite plasma membrane via the apical duct(s). A concentration gradient of AMA-1 would then be expected from diffusion in the merozoite plasma membrane from apex to basal pole, and the presence of such a gradient is supported by some immuno-EM evidence (33). The present results would be consistent with a model for invasion in which the early oscillations of the merozoite and RBC seen in random attachment lead to RBC contact with a merozoite membrane region where some AMA-1 is present. Making and breaking of AMA-1-RBC bonds would then tend to rotate the merozoite "up" this concentration gradient and so establish apical reorientation. Conjecturally, it is also possible that the final processing of AMA-1 may occur in order to promote reorientation, for example by changing the affinity of AMA-1 for an RBC ligand. An analogue of this is seen in the changing affinity of processed *Toxoplasma gondii* MIC2 (10). Neither shed 48- and 44-kDa proteolytic fragments of PfAMA-1 (21) nor an *Escherichia coli*-derived and refolded recombinant PfAMA-1 complete ectodomain (15, 18) have shown RBC binding activity, but when expressed on COS cells, the full-length *P. yoelii* molecule bound RBC, but less well than did contiguous domains 1 and

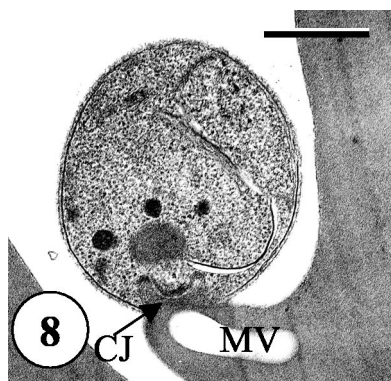


FIG. 8. An example of close junctional (CJ) attachment between the apex of a merozoite and an RBC during normal invasion, with the formation of a membranous vacuole (MV) in the RBC at the merozoite apex. Scale bar, 0.5  $\mu$ m.

2 (17). The affinity for RBC of AMA-1 and its processed fragments may deserve further close scrutiny in the light of the present results.

It is an alternative possibility that under normal circumstances reorientation is brought about randomly by the observed dynamic wrapping of the RBC around the merozoite, which is independent of AMA-1, and that AMA-1 is necessary for the final stabilization of the merozoite's contact when it has arrived at the correct orientation for junction formation to proceed. The existence of membranous blebs beneath some RBC surfaces, which are typical of early normal invasion, suggests that some transient apical contacts did occur in these experiments and were attended by some membranous secretion, although insufficient to initiate full invasion. Thus, AMA-1 may act together with erythrocyte binding proteins (EBPs) (2), also initially localized to the micronemes, to form part of the apical close junction itself and thus may be necessary (but not sufficient) to create the prolonged intimate contact between the RBC membrane and merozoite apex which is required to allow completion of invasion.

The formation of the junction by *P. knowlesi* is held to be dependent on Duffy binding proteins, members of the conserved EBP family (2). When *P. knowlesi* merozoites interact with Duffy blood group-negative human RBC, invasion fails as no junction is formed (23). The EBPs involved have been investigated, and functional domains have been described (see for example, reference 31). Since EBPs are also micronemal proteins (1), one must consider two further possibilities. Incubation with anti-AMA-1 antibody could inhibit invasion indirectly by impeding the extrusion of these micronemal antigens if antigen-antibody complexes were to form and plug the opening of the apical exit duct(s), or anti-AMA-1 antibody could sterically hinder a separate but essential receptor-ligand interaction. We have not rigorously excluded such effects in these experiments, but we consider these modes of action unlikely for the following reasons. First, the MAb used here is more effective on a molar basis in blocking invasion when used as an Fab fragment than as an intact antibody (34). In that monovalent form it could not cross-link antigen, so inhibition could hardly be due to duct blockage. Equally, if steric hindrance of a receptor-ligand interaction unrelated to AMA-1 were caused by antibody to AMA-1, the effect of the larger whole antibody molecule might be expected to be greater than the effect of Fab and not less as was found (34). Secondly, it is likely that *P. knowlesi* merozoites emerging from schizonts already have surface-distributed AMA-1, as disclosed by surface radioiodination, and that before schizont rupture this is unavailable to the MAb (14). Again, this is inconsistent with inhibition by duct blockage. Finally, cultures of rupturing *P. knowlesi* schizonts, free of significant numbers of contaminant normal RBC, show both the processed fragments of AMA-1 in their supernatants (14). These findings support the view that AMA-1 is already extracellular somewhat before (and independently of) the invasion event, and hence, again, that micronemal secretion of AMA-1 has occurred before the observed point of action of the MAb.

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